PLANT-INCORPORATED PROTECTANTS BASED ON PLANT VIRAL COAT PROTEINS

Introduction

A plant-incorporated protectant (PIP) is a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof[1], and the genetic material necessary for production of such a pesticidal substance. The term includes both active and inert ingredients. PIPs are regulated as pesticides by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) because they meet the FIFRA definition of a pesticide, being intended for preventing, destroying, repelling, or mitigating a pest.

PIPs may be genetically engineered into plants[2]. Some specific genetic sequences, when incorporated into a plant's genome, can endow the plant with the ability to resist damage from certain pests. Plant virus coat protein PIPs (PVCP-PIPs) are PIPs in which the inserted genetic material is derived from a plant virus sequence that encodes a coat protein. Plant virus coat proteins encapsidate the viral nucleic acid and are known to have a role in nearly every stage of viral infection including replication, movement throughout an infected plant, and transport from plant to plant. Incorporation of plant viral coat protein (PVCP) gene sequences into plant genomes has been found to confer resistance to the virus from which it was derived, and often to related viruses (OECD Environment Directorate 1996).

Background

In 1994 EPA published its policy regarding regulation of PIPs (then called plant-pesticides) under FIFRA. Recognizing the breadth of the definition of a PIP and that some PIPs present a lower overall risk and thus may not require regulation, the Agency proposed several exemptions to regulation. Included were two options to exempt PVCP-PIPs. A full categorical exemption from regulation under FIFRA was proposed for PVCP-PIPs based on the rationale that they generally pose a low probability of risk to human health or the environment. However, recognizing that other plants could acquire the virus resistance through hybridization with a transgenic plant, an

[1] The phrase "or produce thereof" is included in the definition of a PIP to make it clear that pesticidal substances active in the fruit or other plant product for pesticidal purposes are also considered to be PIPs.
[2] PIPs may also be found naturally-occurring in plants or may be introduced through conventional breeding. However, the focus here is on PIPs, specifically those based on viral coat proteins, that are introduced into plants through genetic engineering.

alternative to a full categorical exemption was also proposed to promote full discussion of this issue. Under this alternative exemption option, the Agency defined a set of criteria to identify those viral coat protein/plant combinations with the lowest potential to confer selective advantage on wild or weedy plant relatives. Only those PVCP-PIPs so identified would have been exempt from regulation. The 1994 policy statement described this alternative exemption as follows:

> Coat proteins from plant viruses [would be exempt] if the genetic material necessary to produce a coat protein is introduced into a plant's genome and the plant has at least one of the following characteristics:
>
> (1) The plant has no wild relatives in the United States with which it can successfully exchange genetic material, i.e., corn, tomato, potato, soybean, or any other plant species that EPA has determined has no sexually compatible wild relatives in the United States.
>
> (2) It has been demonstrated to EPA that the plant is incapable of successful genetic exchange with any existing wild relatives (e.g., through male sterility, self-pollination).
>
> (3) If the plant can successfully exchange genetic material with wild relatives, it has been empirically demonstrated to EPA that existing wild relatives are resistant or tolerant to the virus from which the coat protein is derived or that no selective pressure is exerted by the virus in natural populations.

Some proposals in the 1994 policy statement were finalized in 2001. However, neither the full nor alternative exemption for PVCP-PIPs was finalized, in part because more recent information had questioned the idea that PVCP-PIPs would generally pose a low probability of risk and would not pose unreasonable adverse effects to human health or the environment in the absence of any regulation under FIFRA. For example, the 2000 National Research Council (NRC) report, Genetically Modified Pest-Protected Plants, recommended that,

> "EPA should not categorically exempt viral coat proteins from regulation under FIFRA. Rather, EPA should adopt an approach, such as the agency's alternative proposal…, that allows the agency to consider the gene transfer risks associated with the introduction of viral coat proteins to plants."

In addition to the risks associated with gene transfer, the 2000 NRC report considered other concerns associated with the use of PVCP-PIPs, including the potential for adverse effects following recombination, heterologous encapsidation, and synergy. Although the report concluded that, "[m]ost virus-derived resistance genes are unlikely to present unusual or unmanageable problems that differ from those associated with traditional breeding for virus resistance," neither formulation of the exemption proposed in 1994 contained provisions that would have enabled EPA to ensure that the

risk management strategies suggested by the NRC would be implemented, e.g., elimination of specific sequences to limit the potential for recombination.

In 2001 the Agency reopened the comment period on the PVCP-PIP exemption proposals and sought advice from the public on how best to proceed. EPA is in the process of reevaluating how PVCP-PIPs will be treated by the Agency in light of comments and advice it received. The Agency is reconsidering the scientific merit and feasibility of exempting a subset of PVCP-PIPs from regulation under FIFRA as one of several regulatory options. Potential conditions intended to reduce the risks associated with the use of PVCP-PIPs are included in the questions to the panel to provide a structure that may facilitate discussion of environmental concerns raised with regard to commercial use of PVCP-PIPs.

Charge to the SAP

EPA requests that the Scientific Advisory Panel provide scientific advice to assist EPA in its evaluation of plant-incorporated protectants (PIPs) based on plant virus coat proteins (PVCP-PIPs). During the public comment periods for PVCP-PIPs in 1994 and again in 2001, the Agency received comments from the scientific community that covered viewpoints ranging from support for a full categorical exemption to opposition to any exemption procedure for PVCP-PIPs. These comments highlighted several areas of scientific uncertainty that are addressed in our questions to the panel. The Agency asks for the panel's scientific advice in three areas: gene flow, viral interactions, and other issues. EPA seeks the assistance of the SAP in evaluating whether gene flow and viral interactions may pose environmental hazards, and if so, whether these concerns could be mitigated under certain circumstances. EPA seeks the advice of the SAP to ensure that PVCP-PIPs are appropriately described and to develop a more complete technical record.

Charge – Gene Flow

Gene flow from plants containing PVCP-PIPs is a potential concern to the extent that new genes conferring virus resistance may be introduced into wild or weedy relatives with the potential either to release them from growth and/or reproduction constraints imposed by naturally occurring virus(es) or conversely to decrease their fitness through changes in gene expression. These relatives of transgenic plants may then have the potential for increased weediness and/or the ability to outcompete other species. Conversely, plants also may become less competitive.

A broad review of hybridization between plants and their wild relatives, including the potential for and possible implications of the movement of transgenes, was recently published (Ellstrand 2003), and the issue of introgression from genetically modified crops to their wild relatives was considered in a recent review (Stewart et al. 2003). In addition, several scientific review panels have recently analyzed the general issue of gene flow with particular attention to the regulatory issues facing both U.S. and foreign government agencies (National Research Council 2000; GM Science Review Panel 2003)[3]. The potential effects of gene flow to wild relatives specifically from plants containing PVCP-PIPs has been addressed in a few experimental studies (Bartsch et al.

[3] The UK Government, the Scottish Executive, the National Assembly for Wales and the administration in Northern Ireland have been promoting a national dialogue on genetic modification (GM) issues. One part of this was a review of the science of GM, led by Sir David King (the Government's Chief Scientific Adviser) working with Professor Howard Dalton (the Chief Scientific Adviser to the Secretary of State for the Environment, Food and Rural Affairs), with independent advice from the Food Standards Agency.

1996; Bartsch et al. 2001; Spencer & Snow 2001; Ilardi & Barba 2001; Fuchs et al. 2004a; Fuchs et al. 2004b). This issue is considered in greater depth in several reviews (Bartsch 1997; Power 2002; Tepfer 2002). The theoretical possibility that gene flow from plants containing PVCP-PIPs to wild or weedy relatives could, in some instances, lead to environmental impacts is widely accepted. However, empirical or observational data on the likelihood or magnitude of such impacts are lacking. Consequently EPA is seeking the assistance of the SAP to attempt to reach a better understanding of the circumstances in which the flow of PVCP-PIPs from transgenic plants to wild or weedy relatives could occur and the potential for adverse impacts from such gene flow.

Questions – gene flow

Concerns about the transfer of virus resistance to wild or weedy relatives include the assumption that such resistance might confer a selective advantage to a wild or weedy relative that could increase its competitive ability and potential to become weedy or invasive. The Agency would like the panel to consider the evidence supporting this assumption.

1. **What scientific evidence supports or refutes the idea that plant viruses have significant effects on reproduction, survival, and growth of plant populations in natural settings? Is there scientific evidence that plant populations freed from viral pressure could have increased competitive ability leading to changes in plant population dynamics?**

In the 1994 proposal, EPA identified tomato, potato, soybean, and corn as having no wild relatives in the United States with which they can successfully exchange genetic material, although the 2002 NRC report , Environmental Effects of Transgenic Plants, suggests that corn and its wild relative Eastern gamagrass (*Tripsacum dactyloides*) might produce hybrids with some fertility at a very low frequency in the United States.

2. **Please comment on the validity of the Agency list of crops that have no wild or weedy relatives in the United States with which they can produce viable hybrids in nature (i.e., tomato, potato, soybean, and corn).**
3. **Please identify other crops that have no wild or weedy relatives in the United States with which they can produce viable hybrids in nature, e.g., papaya, peanut, and/or chickpea.**

The Agency anticipates the need to evaluate data addressing whether transgenic plant species are capable of genetic exchange with wild or weedy plant relatives. In general, EPA is focused on the potential for genetic exchange that can occur in the field. However, evaluations of the potential for genetic exchange are likely to include laboratory studies that are not necessarily an accurate indicator of plants' ability to exchange genetic material outside the lab.

4. **What laboratory techniques used to achieve genetic exchange between species (e.g., embryo rescue, use of intermediate bridging crosses, protoplast fusion) are *not* indicative of possible genetic exchange between these species in the field? Conversely, what techniques, if any, used in laboratory or greenhouse experiments provide the most reliable indication of ability to hybridize in the field?**

EPA recognizes that it may be possible to genetically engineer a plant such that concerns about gene flow to wild or weedy relatives are significantly reduced. However, according to the 2004 NRC report, Biological Confinement of Genetically Engineered Organisms, current techniques for bioconfinement (e.g., sterile triploids, male sterility) are imperfect and are not guaranteed to eliminate entirely gene flow to existing wild relatives. Recent modeling studies suggest imperfections in bioconfinement could result in significant levels of gene introgression in compatible plant relatives over a period of decades (Haygood et al. 2004).

5. **Given that current bioconfinement techniques are not 100% effective, what would the environmental implications be of extremely low transfer rates of virus-resistance genes over time?**

EPA recognizes that concerns about gene flow to wild or weedy relatives may be ameliorated if the introduced virus-resistance trait would give little or no selective advantage to the recipient plant, as would occur if the plant were already tolerant or resistant[4] to the virus to which resistance is conferred. It is obvious that such resistance does exist in some populations because traditional breeding for resistance relies on finding a source of resistance within related cultivated species, old varieties, or wild species (Khetarpal et al. 1998).

6. **Please comment on the prevalence of tolerance and/or resistance to viruses in wild relatives of crops.**

[4] See Appendix for definitions.

7. **Please specify techniques that do or do not provide measures of tolerance and/or resistance that are relevant to field conditions.**
8. **How do environmental or other factors (e.g., temporal variations) affect tolerance and/or resistance? Given the expected variability, what measures of tolerance and/or resistance would be reliable?**
9. **What would be the ecological significance if a plant population acquired a small increase in viral tolerance and/or resistance above a naturally-occurring level?**

Based on the hypothesis that concerns about the consequences of gene flow to a wild or weedy relative in the United States may be negligible in certain cases, the Agency is considering whether there are mechanisms to adequately address concerns associated with gene flow so that certain types of VCPs would be of such low risk that they would not need to be regulated by EPA. Below are examples of three conditions (modified from those proposed in 1994) that are intended to significantly reduce any potential adverse effects of gene flow with plants containing a PVCP-PIP.

(1) The plant into which the PVCP-PIP has been inserted has no wild or weedy relatives in the United States with which it can produce viable hybrids in nature, e.g., corn, tomato, potato, or soybean; or

(2) Genetic exchange between the plant into which the PVCP-PIP has been inserted and any existing wild or weedy relatives is substantially reduced by modifying the plant with a scientifically documented method (e.g., through male sterility); or

(3) It has been empirically demonstrated that all existing wild or weedy relatives in the United States with which the plant can produce a viable hybrid are tolerant or resistant to the virus from which the coat protein is derived.

10. **Please comment on how necessary and/or sufficient these conditions are to minimize the potential for the PVCP-PIP to harm the environment through gene flow from the plant containing the PVCP-PIP to wild or weedy relatives. Would any other conditions work as well or better?**

Charge – Viral Interactions

Interactions between introduced plant virus sequences and other invading viruses in transgenic plants (e.g., during recombination or heterologous encapsidation) may be a concern to the extent that such events may increase in frequency or be unlike those expected to occur in nature. It has been hypothesized that such events could lead to the creation of viruses with new disease states or transmission properties. The Agency is evaluating the circumstances that might increase the potential for such events to occur

and the potential environmental consequences of novel viral interactions[5] in light of the 2000 NRC report which stated that, "[m]ost virus-derived resistance genes are unlikely to present unusual or unmanageable problems that differ from those associated with traditional breeding for virus resistance." The report went on to suggest that risks might be managed by particular ways of engineering transgenes[6]. However, under either of the 1994 proposed exemptions, the Agency would be unable to ensure that such strategies were implemented. The Agency's literature review, "Viral interactions in viral coat protein transgenic plants," discusses possible ways of managing these potential risks in detail.

Questions – viral interactions

Viral interactions may occur in natural, mixed infections which are common in plants. Hypothetical concerns related to potential adverse effects resulting from viral interactions between infecting viruses and PVCP-PIPs in transgenic plants may be attributed to opportunities for interactions not expected to occur in nature. EPA is interested in evaluating the significance of *novel* viral interactions involving a viral transgene[7].

11. To what extent are *novel* viral interactions (e.g., recombination, heterologous encapsidation) involving a viral transgene an environmental concern?

Mixed viral infections can be extremely common in crops and other plants. However, scientific uncertainty exists as to whether recombination and heterologous encapsidation would occur more or less frequently in the case of a viral transgene and an infecting virus interaction as compared to such interactions in mixed infections of a transgenic plant's non-bioengineered counterpart[8].

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[5] See Appendix for definition of novel viral interactions.

[6] The report says, "[c]an transgenes be engineered to reduce or eliminate the risk that recombination will spawn new pathogens? Evidence suggests that elimination of genome replication-control sequences from transgenes can limit recombination and therefore risk... Furthermore, strategies to produce resistance-mediating transgenes that encode nonfunction proteins or no protein can be used effectively against viruses. For example, resistant plants that express nontranslatable RNA can confer immunity through induction of post-transcriptional gene silencing…" (National Research Council 2000; pg. 94)

[7] See EPA literature review "Viral Interactions in Viral Coat Protein Transgenic Plants" pp. 15-16.

[8] See EPA literature review "Viral Interactions in Viral Coat Protein Transgenic Plants" pp. 14-15.

12. **What conclusions can be drawn as to whether the likelihood of recombination and/or heterologous encapsidation would be increased or decreased in a transgenic plant compared to its non-bioengineered counterpart?**

A number of methods for reducing the frequency of recombination and heterologous encapsidation have been identified. While the effectiveness of these techniques has been verified for particular cases, their applicability to all PVCP-PIPs is unclear. Recognizing that it would be difficult for a product developer to measure rates of recombination, heterologous encapsidation, or vector transmission under field conditions, EPA is considering whether it would be necessary to verify that such methods worked in any particular instance by measuring rates in modified versus unmodified plants.

13. **How effective is deleting the 3′ untranslated region of the PVCP gene as a method for reducing the frequency of recombination in the region of the PVCP gene? Is this method universally applicable to all potential PVCP-PIP constructs? Would any other methods work as well or better? Which methods are sufficiently effective and reproducible such that actual measurement of rates to verify rate reduction would be unnecessary?**
14. **Are any methods for inhibiting heterologous encapsidation or transmission by insect vectors universally applicable to all PVCP-PIPs? Which methods are sufficiently effective and reproducible such that actual measurement of rates to verify rate reduction would be unnecessary?**
15. **How technically feasible would it be to measure rates of recombination, heterologous encapsidation, and vector transmission in PVCP-PIP transgenic plants in order to show that rates are reduced?**

EPA recognizes that scientific disagreement exists as to the likelihood of environmental impacts due to novel viral interactions in transgenic plants modified with PVCP-PIPs. The Agency is considering whether there are available mechanisms to adequately address concerns associated with novel viral interactions so that certain types of PVCP-PIPs would be of such low risk that they would not need to be regulated by EPA. Below are examples of conditions that might significantly reduce either the novelty [(1) and (2)] or frequency [(3) and (4)] of viral interactions in PVCP-PIP transgenic plants.

(1) The genetic material of the PVCP-PIP is translated and/or transcribed in the same cells, tissues, and developmental stages naturally infected[9] by every virus from which any segment of a coat protein gene used in the PVCP-PIP was derived.

(2) The genetic material of the PVCP-PIP contains coat protein genes or segments of coat protein genes from viruses established throughout the regions where the crop is planted in the United States and that naturally infect the crop into which the genes have been inserted.

(3) The PVCP-PIP has been modified by a method scientifically documented to minimize recombination, (e.g., deletion of the 3' untranslated region of the coat protein gene).

(4) The PVCP-PIP has been modified by a method scientifically documented to minimize heterologous encapsidation or vector transmission, or there is minimal potential for heterologous encapsidation because no protein from the introduced PVCP-PIP is produced in the transgenic plant or this virus does not participate in heterologous encapsidation in nature.

16. Please comment on how necessary and/or sufficient each of these conditions is to minimize the potential for novel viral interactions. Please address specifically what combination would be most effective or what conditions could be modified, added, or deleted to ensure that potential consequences of novel viral interactions in PVCP-PIP transgenic plants are minimized.

Other questions

In 1994 EPA proposed exempting plant viral coat proteins from the requirement of a food tolerance under the Federal Food, Drug, and Cosmetic Act[10] based on rationale that (1) virus infected plants have always been a part of the human and domestic animal food supply and (2) plant viruses have never been shown to be infectious to humans or mammals. The safety of consuming plant virus genes has been supported by experimental work (Chen et al. 2003; Rogan et al. 2000; Shinmoto et al. 1995) and expert consultations including the 2000 NRC report which concluded that, "viral coat proteins in transgenic pest-protected plants are not expected to jeopardize human health because consumers already ingest these compounds in nontransgenic food." However, EPA recognizes that PVCP-PIP developers may wish to modify the PVCP-PIP construct and that some methods of mitigating potential risks associated with recombination and heterologous encapsidation might actually require them to do so. Such modifications

[9] See Appendix for definition of naturally infects.

[10] EPA determines whether limits (tolerances) should be set on the amount of residues of PVCP-PIPs in food derived from the improved plant. When there is substantial information indicating safety and history of safe use, the developer may request an exemption from the requirement of a tolerance. Although a general tolerance exemption for all PVCP-PIPs has not been finalized, tolerance exemptions for specific PVCP-PIPs have been . They can be found in 40 CFR Part 180.1182 (potato), 180.1184 (watermelon), 180.1185 (papaya), and 180.1186 (cucumber).

might result in changes to the protein(s) produced thus creating potential food safety concerns, e.g., inadvertent production of new toxins or allergens (Day 1996). Modifications of the construct and alteration of the proteins produced creates the potential for health impacts on non-target species as well as humans.

17. **To what degree and in what ways might a PVCP gene be modified (e.g., through truncations, deletions, insertions, or point mutations) while still retaining scientific support for the idea that humans have consumed the products of such genes for generations and that such products therefore present no new dietary exposures?**
18. **What are the potential adverse effects, if any, of such modifications on nontarget species (e.g., wildlife and insects that consume the PVCP-PIP)?**

Modifications of the construct may also potentially create the opportunity for novel viral interactions because the inserted virus sequences could be unlike any that occur naturally.

19. **To what degree and in what ways might a PVCP gene be modified (e.g., through truncations, deletions, insertions, or point mutations) before it would be a concern that novel viral interactions due to the modifications could occur because the PVCP gene would be significantly different from any existing in nature?**

The potential risk issues identified in this paper are specific to virus-resistant transgenic plants. However, the Agency recognizes that it may be necessary to evaluate other information related to the PVCP-PIP.

20. **Would any additional requirements related to PVCP-PIP identity and composition (e.g., demonstration that the transgene has been stably inserted) be needed for significant reduction of risks associated with PVCP-PIPs?**
21. **Are there any considerations beyond gene flow, recombination, and heterologous encapsidation as posed in the preceding questions that the Agency should consider in evaluating the risk potential of PVCP-PIPs (e.g., synergy)?**

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Appendix: Definitions

Resistant, when referring to PVCP-PIPs only, means the plant is not infected by or is a non-host of the virus concerned.

Naturally infects means a naturally occurring virus is transmitted to a plant by direct plant-to-plant contact, inanimate objects, or vectors such as pollen, arthropods, nematodes, or fungi; and the virus replicates and moves within the recipient plant. It does not include human intervention, e.g., manual inoculation.

Novel viral interaction means interaction between portions of two or more different viruses (e.g., through recombination or heterologous encapsidation) not expected to occur in a mixed viral infection found in nature.

PVCP-PIP means a plant-incorporated protectant created from the gene, or a segment of the gene, that codes for a coat protein of a virus that naturally infects crop plants.

Tolerant, when referring to PVCP-PIPs only, means the plant is able to sustain the effects of a virus infection with negligible or mild symptom expression and negligible or mild effects on fitness or growth despite the presence of the virus within the host.